A
Synopsis

of thesis entitled

Studies on Pathogenicity and Virulence of *Xanthomonas axonopodis* pv. *punicae*

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Introduction

Pomegranate (*Punica granatum* L.) belongs to the family punicaceae, is one of the favorite table fruit of tropical and subtropical regions. Pomegranate is a high-value plant of fruit from the tropical and sub-tropical areas of India. It is cultivated commercially mainly in the Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan and Tamil Nadu states. Pomegranate, while vulnerable to many diseases. One of the most destructive diseases of *Xanthomonas axonopodis pv. punicae*. The recent bacterial blight outbreaks, especially since 2000, have had an adverse impact on the cultivation of pomegranates in all major states and have led to massive domestic and international market losses. The disease affects all plant components and usually results in the cracking of fruit that is not suitable for use and the market. Hingorani and Mehta (1952) from Delhi, India first reported bacterial blight. The losses from yields in Karnataka have been reported to 60-80% by Chand and Kishun (1991). In the light of the limited available information on bird epidemiology and management strategies, current research has been undertaken in order to evaluate actual damage caused by birth control and study epidemiological disease and spray schedule efficiency to develop appropriate birth control practices.

Pomegranate crop is prone to number of diseases among which bacterial leaf blight caused by *X. axonopodis pv. punicae* (Hingorani and Singh) Vauterin *et al.*, is a serious problem and threat to pomegranate production due to its high epidemic potential. The disease affects all the above ground plant parts including flower, leaves, twigs, stem, buds and fruits, but it is more destructive when fruits are infected. The disease is reported to cause 30-50 per cent losses on an average. However, under favourable environmental conditions 80-100 per cent losses are reported. All the commercial grown cultivars are susceptible to this disease. The disease spread easily in the orchard through plant-to-plant contact, rains, runoff water or spray water splashes, windblown rain splashes, person handling the plant, contaminated tools and insects. It has been seen that under favourable temperature and humid conditions, disease incidence increases after every shower (Anonymous, 2008, 2012).

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis pv. punicae* has become the major threat for pomegranate cultivation in Maharashtra, Karnataka and Andhra Pradesh states. The pathogen attacks all the above ground parts of the plant including fruits, leading to considerable reduction in fruit quality and market value.

The infection starts with the appearance of minute dark colored, irregular spots. As the severity increases the leaves drop. The fruit infection is the most damaging stage resulting in

dark brown irregular slightly raised spots with oily appearance, further it leads to nodal blight on stem portion (stem girdling) (Suria Chandra selvan *et al.*,1993).

X. axonopodis pv. punicae is a bacterial pathogen that causes serious blight disease of pomegranate. The disease was first reported in India where it is a major threat to pomegranate cultivation due to lack of effective control measures. The blight can cause 50 to 100% production loss depending on disease severity. In Australia, pomegranate is one of the emerging industries that has high potential to expand in coming years. The causal agent of this deadly pomegranate disease is not yet reported in Australia. But it has the potential to enter, establish and trigger significant damage to the newly growing industry. Till now *X. axonopodis pv. punicae* is reported in a few states of India only and pomegranate is being claimed as the only host.

The pathogen attacks all the above ground plant parts including fruit splitting that results in huge yield and market losses. The initial symptoms appear as water-soaked translucent irregular to circular minute black spots on leaves. Gradually the centre of the spots become necrotic and turn dark brown. In severe cases, spots coalesce and produce a large patch that may result in shedding of infected leaves. Dark spot symptoms develop on stem nodes, start off cracking and easily break off the branches. Bacterial ooze is used for chemical diagnoses of the disease. The disease initiates through wounds and its spread is influenced by increased day temperature, low humidity and rain. The bacteria overwinters in infected plant leaves, stems and fruits. Rain splash, insects and contaminated pruning tools helping spreading the disease locally. International trade and tourism are responsible for long distance spread via infected plants, twigs, and fruits.

The disease affects all plant parts and normally results in cracking of fruits which are rendered unfit for consumption and the market. Bacterial blight was first reported by Hingorani and Mehta (1952) from Delhi, India. Chand and Kishun (1991) reported 60-80% yield losses due to blight epidemics in Karnataka. In view of scant information available on blight epidemiology and management strategies, the present work will help to assess the actual damage due to blight, to study disease epidemiology and factors governing the virulence of the pathogen and subsequently to develop suitable blight management practices.

Review of literature & development in the subject:

Pomegranate (*Punica granatum* L.) is a high value horticultural crop of subtropical and tropical regions of the world especially cultivated in Iran, India, Spain, Afghanistan and South Africa. At global level, India and Iran are the major producers of pomegranate with collective contribution of about 85% of the total production. In 2014-15, India produced 17.89 lakh tons

pomegranate (www.nhb. gov.in). The major production constraint in pomegranate is bacterial blight disease that takes heavy toll of crop and reduces the marketability of fruits (Chand and Kishun, 1991; Ravikumar *et al.,* 2009; Sharma *et al.,*2012a). Bacterial blight was first reported in India from Delhi by Hingorani and Mehta (1952). Subsequently, it was reported from Karnataka (Hingorani and Singh, 1959), Himachal Pradesh (Sohi et al., 1964), Haryana (Kanwar, 1976) and Maharashtra (Dhandar*et al.,* 2004; Sharma *et al.,*2008, 2009, 2010, 2012a). The disease outbreak had reached an epiphytotic level in several parts of central India. The disease is reported from other countries such as Pakistan (Akhtar and Bhatti, 1992), South Africa (Petersen *et al.,*2010) and very recently in Turkey (Icoz*et al.,*2014)

The causal organism of blight is *Xanthomonas axonopodis pv. punicae* (Hingorani and Singh) Vauterin, Hoste, Kersters and Swings (Hingorani and Mehta, 1952; Vauterin et al, 1995). The plant is susceptible to blight during all stages of growth and results in huge economic loss. Bacterial blight primarily affects the above ground plant parts, especially leaves, twigs and fruits. While the leaves show early water soaked lesions to late necrotic blighting, the fruits show isolated or coalesced water soaked lesions followed by necrosis with small cracks and splitting of the entire fruit (Petersen *et al.*, 2010). Stems show lesions around nodes or injuries, forming cankers in later stages. Suspected symptoms on floral parts have also been reported (Chand and Kishun, 1991; Rani *et al.,*2001). It is also presumed that the stem canker could be an outcome of systemic spread of bacterium from leaf (Chand and Kishun, 1993). Although reported by Chand and Kishun (1991) and Rani *et al.,*(2001) attempts to reproduce field symptoms of blight on detached leaves, twigs and fruits were unsuccessful in the artificial inoculations.

Bacterial blight caused by *X. axonopodis pv. punicae* is one of the important production constraints of pomegranate in India. The disease is also recently reported in South Africa (Petersen *et al.,*2010) and Turkey (Icoz*et al.,*2014). In India, the disease is widely prevalent in the states of Maharashtra, Karnataka, Andhra Pradesh, Himachal Pradesh and Rajasthan causing severe quantitative and qualitative losses. The disease is most damaging when the fruits are affected.

Variety of symptoms ranging from water-soaked lesions to dark brown to black necrotic lesions on leaves, fruits, and stem nodes were recorded. The lesions enlarged, co-alesced and culminated in characteristic blighted appearance when the disease advanced with time. Bacterial blight symptoms have been earlier reported by several authors (Hingorani and Mehta, 1952; Hingorani and Singh, 1959; Sohi*et al.,*1964; Kanwar, 1976; Kishun, 1993; Rani and Verma, 2002). Besides commonly reported blight symptoms, we report here additional symptoms observed under specific environmental conditions. Extended blight lesions on leaf blade along

the margins or tip downward were observed especially during high humid conditions as well as heavy dew nights, indicating hydathode entry of the pathogen. Bright yellowing of leaves and necrosis above the nodal canker on young twigs was so characteristic that the affected plants could easily be spotted in orchards from distance. On fruits, in addition to lesions and cracking, other pathological symptoms like deposition of shining white encrustation and hanging of bacterial ooze on lesions was also observed during high humid conditions. Floral blight symptoms have been reported by several researchers in the past (Chand and Kishun, 1991; Rani *et al.*,2001).

Objectives of Research:

1. To isolate *Xanthomonas axonopodis pv. punicae* species from different plant (host range) from different locations in Maharashtra.
2. To characterize of *Xanthomonas axonopodis pv. punicae* species by biochemical physiological and molecular methods.
3. To study the traits governing pathogenicity among *Xanthomonas axonopodis pv. punicae* pathovars.
4. To study Interaction studies of plants and pathogenic *Xanthomonas axonopodis pv. punicae* strains.
5. To determine genetic basis of various virulence factors like EPS, LPS, DE, Adhesin, virulence related proteins etc.

Methodology to be adopted:

1) Survey of bacterial disease on pomegranate & collection of plant material from infected pomegranate crop.
2) Diseased plant samples will be collected various field in the regions and Bacteria will be isolated and purified on Nutrient starch cycloheximide antibiotic agar.
3) After presumptive species identification based on yellow pigmented colonies further biochemical and physiological characterization will be done
4) Each strain will be tested for pathogenicity on host plant by needle/syringe inoculation techniques, only virulent bacteria will be maintained for further study.
5) Pathogenic bacterial isolates from infected pomegranate will be maintained on Nutrient Agar /Yeast Dextrose Carbonate (YDC) medium etc. and further maintenance of bacterial culture will be done.

6) Hypersensitivity test will be carried out.
7) DNA will be isolated from selected strains by using standard procedure. Purity of DNA will be check.
8) Various virulence factors like EPS, LPS, DE, Adhesin, virulence related proteins etc. will be studied.
9) Genetic basis of various virulence factors like EPS, LPS, DE, Adhesin, virulence related proteins etc. will be studied.
10) Analysis of results obtained from these analyses will be done.

Importance of study / Society application:

International status:

Pomegranate (*Punica granatum* L.) is a woody perennial fruit crop grown in arid zones of India, the state of Maharashtra being considered as pomegranate basket contributes to 70% of the total area followed by Karnataka and Andhra Pradesh. Protection of crops against bacterial disease is an important issue in agricultural production. Pomegranate production in India is severely hampered by the high incidence of bacterial blight disease caused by *Xanthomonas axonopodis pv. punicae* is air borne, the conventional ways and means of controlling this disease have failed. Evolving a resistant genotype using resistant variety through conventional breeding may be a way out but it is a time-consuming process. Transgenic approach appears to be promising to minimize the losses caused by disease.

Xanthomonas axonopodis pv. punicae is one of the most destructive diseases. The recent outbreaks of bacterial blight, particularly since the year 2000, have adversely affected pomegranate cultivation in all major states and resulted in enormous losses both in the domestic and international markets.

Hosamani *et al.,* (2018) Efficient protocols were developed to get healthy and well-formed plants from juvenile and mature-origin explants of the pomegranate cv. ‘Bhagwa’ and transformants with PFLP gene. One of the strategies to lead plants become resistant against bacterial pathogens is employing a transgene, like plant ferredoxin-like protein (PFLP). Different treatment combinations of hormonal concentrations were taken for leaf, petal, nodes and cotyledonary explants to standardize an efficient in vitro regeneration protocol and find out the best treatment for faster regeneration. Agrobacterium tumefaciens carrying gene pCAMBIA construct with the constitutive CaMV35S promoter, PFLP gene, terminator and nptII selectable marker (Kanamycin resistance), was used for transformation of explants. Putative transformants

were identified on selection medium containing kanamycin at different concentration. Integration of transgene and expression at various levels were confirmed using PCR. Of 4 putative transformants analyzed, three plants showed amplification for PFLP gene specific primer. (Kumar *et al.,* 2021)

National Status:

In a study, isolation of *Xanthomonas axonopodis pv. punicae* from pomegranate growing at Solapur, Latur, Osmanabad and Jalna districts of Maharashtra state showing seven isolates showed characters of *X. axonopodis pv. punicae* with yellow mucoid shining colonies were reported. (Pawar*et al.,* 2021)

Hunjan *et al.,* (2019) reported extracellular polysachharides (EPS) abundantly produced by several plant pathogenic bacteria especially by those inhabiting the phyllosphere. Besides their role in protecting the bacteria from hostile environmental conditions, they play a diverse role in bacterial aggregation, and hence virulence. EPS from twenty-seven isolates of *Xanthomonas axonopodis pv. punicae* were quantified and correlated with aggressiveness of the pathogen. The possible role of EPS in facilitating the bacteria to establish itself during early stages of infection.

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis pv. punicae*, have economic importance to reduced crop yield in India. It was revealed that the maximum disease incidence (91%), severity (14.41%) and fruit cracking (12%) was found in cv. Ganesh. Twenty isolates of *Xanthomonas axonopodis pv. punicae* showed different virulent reaction with respect to incubation period, fruit cracking and drooping. It was observed that 9 isolates were highly virulent on Ganesh cultivar. The optimum growth of bacterium was observed at 30°C while 5 and 50°C temperatures did not show any growth on Kings B Medium. (Kumar *et al.*,2018) Although some work has been carried out on the occurrence of this pathogen in India. Molecular characterization using various DNA method has been studied but a little work or no studies is being carried out on various genetic and molecular basis of virulent determinants in *Xanthomonas axonopodis pv. punicae*

Significance of the study

Xanthomonas axonopodis pv. punicae has been a model for studying how bacteria cause plant diseases for almost 60 years, but in many cases, we have relied on research approaches that are akinto shooting in the dark. However, progress in the genomic analysis of the *Xanthomonas*

axonopodis pv. punicae has now shown a bright light on some previously obscure aspects of this pathogen's inner workings. The field now seems prepared to move forward with greater assurance toward fulfilling our dream that basic research on the pathogen can help provide innovative tools and strategies to control *Xanthomonas axonopodis pv. punicae* disease.

A greater understanding of the genomic features of bacteria that make them important for agriculture could improve our ability to predict the utility of these microbes for agriculture. There is no question that the microbial pathogen control is critical for the success, not only of commercially important plants, but for restoring habitat for native plants. A fusion of information about genes and function will increase our ability to prescribe treatments that will have a wide spread effect on various aspects of agriculture.

Proposed work plan / Formulation & structure of study:

First year

1. Collection of diseased samples from field at various locations during the study period.
2. Isolation of *Xanthomonas axonopodis pv. punicae* from diseased plant parts and maintenance of cultures.

Second year:

1. Characterization of isolated strains of *Xanthomonas axonopodis pv. punicae* by nutritional and cultural properties.
2. Standardization of DNA based techniques and 16S rRNA typing for assessment of diversity.

Third year:

1. Characterization of genetic basis of virulence and pathogenicity by isolated strains of *Xanthomonas axonopodis pv. punicae* using standardized DNA based techniques.
2. Deriving relationship various pathogenicity factor in the isolates and disease incidence and identifying genetic basis of these virulence determinants.

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